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Inhibition of Induced Differentiation of C3H/10T 1/2 Clone 8 Mouse Embryo Cells by Tumor Promoters

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Inhibition of Induced Differentiation of C3H/10T½ Clone 8 Mouse Embryo Cells by Tumor Promoters[1]

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ABSTRACT

C3H/10T½ cells were induced to differentiate into muscle cells by treatment with 5-azacytidine, and the effects of tumor promoters, nonpromoters, and inhibitors of tumor promotion on this induced differentiation were investigated. Cell morphology was dramatically changed within 30 min after treatment with phorbol ester-related tumor promoters and mezereine but not with other tumor promoters. There was a good correlation between the tumor-promoting activity of the compounds and their inhibitory action on differentiation except in case of phenobarbital; this promoter of liver carcinogenesis did not inhibit differentiation. Inhibitors of tumor promotion, dexamethasone, fluocinolone acetonide, retinoic acid, and antipain, also inhibited the 5-azacytidine-induced differentiation. When the cells were treated after induction of differentiation with 12-*O*-tetradecanoylphorbol-13-acetate, a potent tumor promoter, and simultaneously with one of those inhibitors of tumor promotion, the inhibitory action of 12-*O*-tetradecanoylphorbol-13-acetate was not affected. From these facts, it is evident that using a single phenomenon produced by one class of tumor promoter as a criterion for screening environmental tumor promoters is not justifiable.

INTRODUCTION

Cancer can be considered as a problem involving disturbances in the regulation of cell proliferation and differentiation (29). Many tumor promoters have a mitogenic action on cells *in vivo* and *in vitro* (3, 43), and normal cells treated with some tumor promoters temporarily mimic in some respects transformed cells (42). In order to study the mechanism of tumor promotion, it is reasonable to determine whether it is associated with the process of dedifferentiation or inhibition of differentiation. There are reports that TPA,[3] a potent tumor promoter (10), inhibits the further differentiation of chick embryo myoblasts and chondroblasts (4, 26), Friend erythroleukemia cells undergoing either spontaneous or induced differentiation (32, 44), differentiation of 3T3 cells to lipocytes (7), differentiation of neuroblastoma cells (14), and differentiation of mouse myeloid cells induced by dexamethasone and a protein inducer obtained from conditioned medium (16). The inhibition of differentiation by TPA and related diterpenes closely correlates with their tumor-promoting activities. However, in a recent publication, Miao *et al.* (22) showed that the inhibitory action of TPA and related compounds on differentiation was not universal since they have both found inhibitory and stimulatory actions of TPA on spontaneous and induced differentiation, using Friend virus-transformed and Rauscher virus-transformed proerythroid cell lines. Huberman and Callahan (13) and Rovera *et al.* (33, 34) demonstrated a stimulation of differentiation of human promyelocytic leukemic cells by TPA and other tumor promoters. This stimulatory activity on differentiation was also closely related to tumor-promoting activities. Thus, phorbol ester tumor promoters both stimulate or inhibit terminal differentiation, depending upon the cell lines used.

In all those cell culture systems, 2-stage transformation was either not possible or has not been shown. In the C3H/10T½ clone 8 mouse embryo cell line (30), we have demonstrated 2-stage oncogenic transformation with polycyclic hydrocarbons (23), UV (25), and X-rays (18) as initiators and TPA and related compounds as promoters. We have also shown in this system that saccharin has promoting activity (24). Thus, these cells appear to be a valid model system to screen various chemicals for tumor-promoting activity and also to study the mechanism of tumor promotion. Jones *et al.* (5, 6, 39) have made the interesting discovery that C3H/10T½ cells differentiate into muscle cells, adipocytes and chondrocytes, following 5-aza-CR treatment. We took advantage of this phenomenon to study the effects of various tumor promoters and nonpromoters on the differentiation of C3H/10T½ cells induced by 5-aza-CR treatment and to determine whether any observed effects could be meaningfully associated with tumor promotion.

MATERIALS AND METHODS

Cell Culture and Media. C3H/10T½ Clone 8 cells were plated in 60-mm plastic culture dishes (1000 cells/dish; Corning Glass Works, Corning, N. Y.) in 5 ml of BME supplemented with 10% heat-inactivated FCS and incubated at 37° in humidified incubators (Forma Scientific, Inc., Marietta, Ohio) in an atmosphere of 5% CO_2 in air. Twenty-four hr after plating, the cells of the experimental group were treated with 5-aza-CR (0.25 to 0.75 μg/ml) in BME + 10% FCS and incubated for 24 hr after which the 5-aza-CR-containing medium was removed; the cells were washed once and fed with normal medium (BME + 10% FCS). The cells were then cultured for 18 to 21 days with twice-weekly medium changes. The tumor promoters and nonpromoters were added 48 hr after washing out the 5-aza-CR (if not otherwise mentioned). At the end of the experiments, the dishes were fixed with 100% methanol and stained wth 7% Giemsa; the differentiated muscle cells which appeared as elongated, multinucleated, often branching, giant cells were counted in each dish under a stereo-zoom microscope. The average number of muscle cells from 6 dishes/experiment and 2 to 4 experiments was calculated for each point.

Chemicals. 5-aza-CR was purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions were made up fresh for

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[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 5-aza-CR, 5-azacytidine; BME, Eagle's basal medium; FCS, fetal calf serum; EGF, epidermal growth factor.

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each experiment in phosphate-buffered saline and sterlized by filtration. Iodoacetic acid, acetic acid, dexamethasone, and Tween 60 were also obtained from Sigma. TPA and phorbol were purchased from Dr. Peter Borchert (University of Minnesota, Minn.). Phorbol dibenzoate, phorbol diacetate, phorbol dibutyrate, 4-α-phorbol didecanoate, fluocinolone acetonide, and mezereine were generous gifts of Dr. T. Slaga (Oak Ridge National Laboratory). A supply of mezereine was also obtained from Dr. L. Diamond (Wistar Institute, Philadelphia, Pa.). Anthralin was a gift from Dr. R. K. Boutwell (McArdle Laboratory for Cancer Research, Madison, Wis); retinoic acid was a gift from Dr. M. B. Sporn (National Cancer Institute, Bethesda, Md.); EGF was kindly supplied by Dr. H. Herschman (University of California, Los Angeles); saccharin was obtained from Dr. D. R. Stoltz (Toxicology Research Division of the Health Protection Branch, Health and Welfare, Ottawa, Ontario, Canada); and antipain was given to us by Dr. W. Troll (New York University Medical Center, N. Y.).

Preliminary cytotoxicity experiments were done with all the above compounds, and with the exception of 5-aza-CR all were used at nontoxic concentrations.

RESULTS

Cell death and differentiation to muscle cell formation following 5-aza-CR treatment varied from batch to batch of 5-aza-CR purchased. Table 1 shows the average number of muscle cells per dish with various concentrations of 5-aza-CR together with its cytotoxicity, as determined by reduction of plating efficiency. At the highest concentration (0.75 μg/ml), the average number of muscle cells per dish was maximal, but this concentration killed about 50% of the cells. Thus, for all subsequent experiments, we chose 5-aza-CR (0.5 μg/ml), which was less toxic and produced sufficient numbers of muscle cells. MCA clone 16, a malignant cell line derived from treating C3H/10T½ cells with 3-methylcholanthrene (30), was less sensitive to 5-aza-CR cytotoxicity and formed a very small number of differentiated cells only at a concentration of 0.75 μg/ml and above. The data are not shown here because it was very difficult to quantitate the number of muscle cells that result from the piled-up growth pattern of this cell line. We restrict the term "differentiated cells" to muscle cells only, as characterized by Constantinides *et al* (6), and we did not identify adipocytes and chondrocytes, which are also produced by 5-aza-CR treatment (39); we also did not investigate the effect of tumor promoters on the formation of those 2 types of differentiated cells.

TPA inhibited the 5-aza-CR-induced differentiation at all concentrations tested (Table 2). In these experiments, we added TPA to the cultures 48 hr after washing the 5-aza-CR from the cells, and inhibition of muscle cell formation was similar at all concentrations of TPA from 0.1 to 1.0 μg/ml. Since we customarily use TPA at 0.1 μg/ml in our research on promotion of transformation (18, 23–25), for all subsequent experiments we used only the concentration of the compounds that we have already demonstrated to have promoting activity on transformation of C3H/10T½ cells; for other compounds, we used concentrations that were nontoxic, *i.e.*, did not reduce the plating efficiency. Table 3 shows the effects on differentiation produced by TPA, other phorbol diesters, and phorbol that were added at various times after washing out 5-aza-CR. When TPA was added 48 hr after 5-aza-CR exposure, there was a maximal inhibition of differentiation with both inducing concentrations of 5-aza-CR; thus, in subsequent experiments we used this timing. Phorbol and 2 of its diesters, 4-α-phorbol didecanoate and phorbol diacetate, produced no inhibition of differentiation; other diesters were significantly inhibitory. The inhibitory activity of these compounds was reflected in the number and size of the differentiated cells; the TPA-treated differentiated cells were thicker and shorter than were the controls (Fig. 1).

Table 1

Induction of muscle cells in C3H/10T½ clone 8 cells by 5-aza-CR Treatment

Concentration of 5-aza-CR (μg/ml)	Plating efficiency (%)	Muscle cells/dish[a]
0	22	0
0.25	18	34 ± 9[b]
0.5	14	160 ± 49
0.75	12	187 ± 12

[a] Average number of C3H/10T½ cells/dish = 7×10^5.
[b] Average ± S.E. of 2 to 4 experiments.

Table 2

Effects of the concentration of TPA on the formation of muscle cells following 5-aza-CR treatment

TPA was added 48 hr after 5-aza-CR was washed from the cells.

Compounds	Relative % of muscle cells
5-aza-CR (0.5 μg/ml)	100
5-aza-CR (0.5 μg/ml) + TPA (0.05 μg/ml)	53 ± 2[a]
5-aza-CR (0.5 μg/ml) + TPA (0.1 μg/ml)	18 ± 4
5-aza-CR (0.5 μg/ml) + TPA (0.25 μg/ml)	16 ± 2
5-aza-CR (0.5 μg/ml) + TPA (0.75 μg/ml)	19 ± 2
5-aza-CR (0.5 μg/ml) + TPA (1 μg/ml)	21 ± 2

[a] Average ± S.E. of 2 to 4 experiments.

Another notable change in the morphology of C3H/10T½ cells was observed within 20 to 30 min of treatment with TPA (the details of this phenomenon are the subject of another publication).[4] The cells became smaller and rounded up with long beady processes (Fig. 2). This effect was reversible and lasted for about 36 hr, after which time the cells returned to normal morphology even in TPA-containing medium. This effect was observable when the cells had been treated previously with 5-aza-CR or other carcinogens and was also found with phorbol dibenzoate, phorbol dibutyrate, and mezereine. Phorbol, phorbol diacetate, 4-α-phorbol didecanoate, and other chemical classes of tumor promoters had no effect on the morphology of the cells (Table 4).

Other chemical classes of active tumor promoters, including anthralin (36), mezereine,[5] and saccharin (11), also have promoting action on the transformation of C3H/10T½ cells initiated by 3-methylcholanthrene or 5-aza-CR (data not shown). Consequently, we were interested to determine whether these compounds would also inhibit the 5-aza-CR-induced differentiation of these cells. As shown in Table 4, they did. Moreover, the tumor promoters, Tween 60 (37) and iodoacetic acid (8), which have not been tested in C3H/10T½ cells for their promoting activity, also inhibited the 5-aza-CR-induced differentiation. Acetic acid, which has very little tumor-promoting action on mouse skin (3) and has not been tested in C3H/10T½ cells, had a stimulatory action on differentiation. EGF, which has tumor-promoting activity on mouse skin (31), showed some

[4] C. Boreiko, S. Mondal, and C. Heidelberger. Effects of 12-*O*-Tetradecanoyl phorbol-13-Acetate (TPA) on the morphology and growth of C3H/10T½ mouse embryo cells, manuscript in preparation.
[5] T. G. Slaga, Oak Ridge National Laboratories, personal communication.

Table 3

Effects of phorbol esters on the formation of muscle cells when added at various times after 5-aza-CR treatment

The results are expressed as relative to the percentage obtained after 5-aza-CR treatment, which was considered as 100%. Values are the average of 2 experiments.

5-aza-CR	0.25 μg/ml (100%)	0.5 μg/ml (100%)	0.75 μg/ml (100%)
+ TPA (0.1 μg/ml) At 0 hr (immediately after washing)	48	46	NT[a]
At 24 hr	30	56	NT
At 48 hr	7.6	18	37
At 72 hr	14	48	NT
At 96 hr	24	60	NT
At 120 hr	15	44	NT
+ Phorbol (0.1 μg/ml) At 48 hr	107	93	105
At 72 hr	NT	138	NT
At 96 hr	93	134	NT
+ 4α-Phorbol didecanoate (0.1 μg/ml) at 48 hr	NT	100	NT
+ Phorbol dibenzoate (0.1 μg/ml) at 48 hr	NT	47	NT
+ Phorbol diacetate (0.1 μg/ml) at 48 hr	NT	84	NT
+ Phorbol dibutyrate (0.1 μg/ml) at 48 hr	NT	67	NT

[a] NT, not tested.

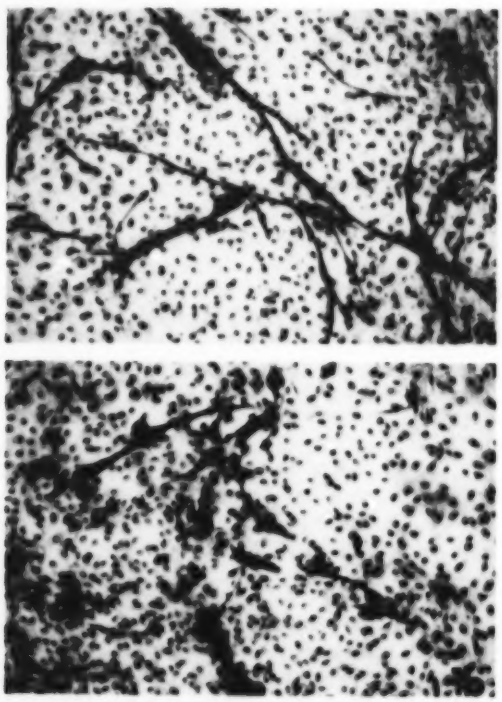

Fig. 1. *A*, fixed and stained culture, showing muscle cell formation on a monolayer of C3H/10T½ cells, 18 days after 5-aza-CR (0.5 μg/ml) treatment. × 400. *B*, fixed and stained TPA-treated culture after 5-aza-CR (0.5 μg/ml) induction, showing muscle cells which are shorter and thicker. × 400.

enhancement of transformation of C3H/10T½ cells after 5-aza-CR initiation (data not shown) and produced marked inhibition of differentiation (Table 4).

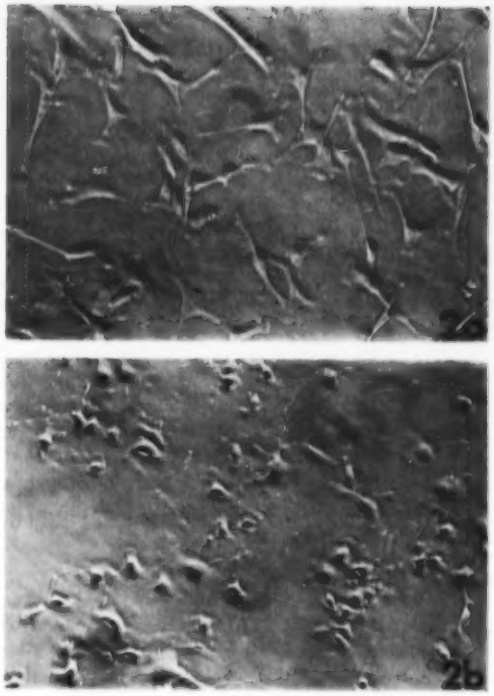

Fig. 2. *A*, phase-contrast view of sparse culture of C3H/10T½ cells. × 1000. *B*, phase-contrast view of C3H/10T½ cells treated with TPA (0.1 μg/ml) 30 min after treatment. × 1000.

The effects of known inhibitors of tumor promotion on the 5-aza-CR-induced differentiation are shown in Table 5. TPA, antipain, retinoic acid, fluocinolone acetonide, and dexamethasone did not by themselves produce any differentiated cells, but they all strongly inhibited the differentiation induced by 5-aza-CR. When these compounds were added to the 5-aza-CR-induced cultures simultaneously with TPA, none of them reversed the inhibitory action of TPA on differentiation, nor was the inhibition produced by TPA significantly affected by the other compounds. EGF alone inhibited differentiation (Table 5); however, when TPA and EGF were added simultaneously to cultures treated with 5-aza-CR, there was no increased inhibition of differentiation.

DISCUSSION

The results presented here show that TPA, phorbol dibutyrate, and phorbol dibenzoate, all of which are active tumor promoters (1, 10), inihibited the 5-aza-CR-induced differentiation of C3H/10T½ cells. The structurally related inactive promoters, phorbol and phorbol diacetate (1), did not exert such an inhibition. This agrees well with the findings from other laboratories in other differentiating systems (4, 7, 14, 22, 44). We reported previously that 4-α-PDD was a weak promoter in C3H/10T½ cells (23) and produced slight inhibition of [^{3}H]thymidine incorporation, which was less than that produced by TPA (28). However, in the present study, 4-α-PDD did not inhibit the 5-aza-CR-induced differentiation, which is in agreement with its *in vivo* inactivity as a promoter. Other chemical classes of tumor promoters including anthralin, mezereine, iodoacetic acid, and Tween 60 also inhibited the 5-aza-CR-

Table 4

Effects of tumor promoters and nonpromoters on C3H/10T½ cells[a]

Compounds[b]	Change in morphology after treatment	Muscle cell formation relative to the % obtained with 5-aza-CR (0.5 μg/ml) treatment (%)[c]	Promoting activity in C3H/10T1/2 cells	Tumor-promoting activity in mice and/or rats[d]
5-aza-CR (0.5 μg/ml)				
+ TPA (0.1 μg/ml)	Changed	19	+	+
+ Anthralin				
0.1 μg/ml	Normal	101	+	+
0.5 μg/ml	Normal	63	+	
+ Mezereine				
1 μg/ml	Changed	39	+	+
2 μg/ml	Changed	20	+	
+ Saccharin				
(100 μg/ml)	Normal	62	+	+
+ EGF (10 ng/ml)	Normal	58	+	+
+ Tween 60 (0.1 μg/ml)	Normal	65	NT[e]	+
+ Acetic acid (0.0125%)	Normal	130	NT	−
+ Iodoacetic acid (0.01 μg/ml)	Normal	14	NT	+
+ Phenobarbital (5 μg/ml)	Normal	100	NT	+

[a] Tumor promoters and nonpromoters were nontoxic to the cells at the concentrations used.
[b] Tumor promoters and nonpromoters were added to the culture 48 hr after the 5-aza-CR (0.5 μg/ml) was washed out.
[c] Average of 2 experiments.
[d] Initiating agents and the doses of tumor promoters were different in animal experiments.
[e] NT, not tested.

Table 5

Effects of inhibitors of tumor promotion[a] *on the inhibition of muscle cell formation by TPA following 5-aza-CR treatment*

Compounds	% of muscle cell formation relative to 5-aza-CR (0.5 μg/ml) treatment[b]
5-Aza-CR (0.5 μg/ml)	100
TPA (0.1 μg/ml)	0
EGF (0.01 μg/ml)	0
Antipain (1 μg/ml)	0
Retinoic acid (0.05 μg/ml)	0
Fluocinolone acetonide (0.5 μg/ml)	0
Dexamethasone (1 μg/ml)	0
5-aza-CR + TPA (0.1 μg/ml)	12
5-aza-CR + antipain (1 μg/ml)	28
5-aza-CR + fluocinolone acetonide (0.5 μg/ml)	5
5-aza-CR + dexamethasone (1 μg/ml)	19
5-aza-CR + retinoic acid (0.05 μg/ml)	10
5-aza-CR + EGF (0.01 μg/ml)	18
5-aza-CR + TPA + dexamethasone (1 μg/ml)	8
5-aza-CR + TPA + retinoic acid (0.05 μg/ml)	14
5-aza-CR + TPA + fluocinolone acetonide (0.5 μg/ml)	13
5-aza-CR + TPA + antipain (1 μg/ml)	16
5-aza-CR + TPA + EGF (0.01 μg/ml)	13

[a] All the compounds were nontoxic to the cells at the concentrations used.
[b] Average of 2 to 3 experiments.

induced differentiation, which the non-tumor promoter acetic acid did not.

Phenobarbital, which acts as a promoter of hepatocarcinogenesis in rats fed 2-acetylaminofluorene (27), did not inhibit differentiation in our system and was also inactive in stimulating the differentiation of human promyelocytic leukemic cells (33). Although anthralin and saccharin were inactive in that system (33), they inhibit 5-aza-CR-induced differentiation and act as promoters in C3H/10T½ cells. Their promoting activity correlates with their inhibition of differentiation in our system. On the other hand, mezereine, which promotes differentiation in the promyelocytic leukemia cells and inhibits differentiation of C3H/10T½ cells, is a promoter in our system. EGF, which is a tumor promoter in mouse skin (31), also inhibited the differentiation, and in a preliminary experiment (data not shown) it also acts as a promoter in C3H/10T½ cells. Thus, there is a good correlation in our system between promoting activity and inhibition of 5-aza-CR-induced differentiation except for phenobarbital, which has not yet been tested as a promoter in these cells.

The morphological changes of C3H/10T½ cells induced by TPA and mezereine were not observed when the cells were treated with other tumor promoters, *i.e.*, anthralin, saccharin, EGF, iodoacetic acid, Tween 60, and phenobarbital. This demonstrates that this change in morphology is a phenomenon related only to TPA and mezereine; thus, it cannot be used as a criterion to screen all classes of tumor promoters distributed in the environment.

Several compounds have been shown to inhibit the tumor-promoting activity of TPA on mouse skin. These include dexamethasone, fluocinolone acetonide (35), and antipain (40). Antipain also inhibits the TPA-induced promotion of transformation of C3H/10T½ cells initiated with X-rays (17), although depending on the time of addition, it inhibited or stimulated the X-ray-induced transformation of these cells (2). Retinoic acid, which maintains differentiation of epithelial tissue and inhibits carcinogenesis (38), also stimulates embryonal carcinoma cells to differentiate (15) and inhibits tumor promotion and ornithine decarboxylase induction produced by TPA in mouse skin (41). Retinoic acid also inhibits the transformation of C3H/10T½ cells by X-rays (9) and chemicals (21).

All the above inhibitors of transformation and tumor promotion strongly inhibited the differentiation of C3H/10T½ cells induced by 5-aza-CR (Table 5). However, when they were added simultaneously with TPA, they produced no further effect. Dexamethasone, retinoic acid, and antipain also did not influence the stimulatory action of TPA on the differentiation of human promyelotic leukemia cells (33). On the other hand, dexamethasone induced the differentiation of mouse myeloid leukemia cells (12). From all the above results, it is clear that even within one test system the effect on differentiation cannot be taken as a criterion for screening all tumor promoters.

Although TPA and EGF both induce plasminogen activator in HeLa cells, when they were added simultaneously at optimal concentrations there was no augmentation or inhibition of the induction (19). On the other hand, TPA inhibits the binding of EGF to cellular receptors of HeLa and xeroderma pigmentosum cells (20). In our experiments, TPA and EGF separately inhibited the 5-aza-CR-induced differentiation, and when they were added together there was no further effect.

When TPA was added to the culture simultaneously with 5-aza-CR and the cultures were washed after 24 hr and then continued in normal medium, there was no inhibition of differentiation. However, when the cells were treated simultaneously with TPA and 5-aza-CR and then the culture was continued with medium containing TPA, there was inhibition of differentiation (data not shown). This shows that TPA has no effect on the cells entering the committed state but acts only after the 24-hr treatment with 5-aza-CR (Table 3), during which the cells become committed to differentiation. As in the case of tumor promotion, where TPA added before the initiator exerts no

promoting activity, prior treatment with TPA has no effect on the 5-aza-CR-induced differentiation of C3H/10T½ cells (data not shown).

Because of the complexity of the pleiotropic effects produced by some tumor promoters, it is difficult to ascertain which of those effects are most directly involved in the mechanism of promotion. It seems likely that promoters of different chemical classes may act through different molecular mechanisms and yet produce the same biological effects.

Since both tumor promoters and inhibitors of tumor promotion cause inhibition as well as stimulation of differentiation in different systems, how can the phenomenon of differentiation be involved in the mechanism of tumor promotion? Clearly, further research is necessary before the involvement of differentiation in the mechanism of tumor promotion can either be established or ruled out.

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